

# A Novel Targeted Therapy of Leydig and Granulosa Cell Tumors through the Luteinizing Hormone Receptor Using a Hecate–Chorionic Gonadotropin $\beta$ Conjugate in Transgenic Mice<sup>1</sup>

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## Abstract

We investigated the antitumoral efficacy, endocrine consequences, and molecular mechanisms underlying cell death induced by the Hecate–chorionic gonadotropin (CG) $\beta$  conjugate, a fusion protein of a 23–amino acid lytic peptide Hecate with a 15–amino acid (81–95) fragment of the human CG $\beta$  chain. Transgenic (TG) mice expressing the inhibin  $\alpha$ -subunit promoter (inh $\alpha$ )/Simian Virus 40 T-antigen (Tag) transgene, developing luteinizing hormone (LH) receptor (R) expressing Leydig and granulosa cell tumors, and wild-type control littermates were treated either with vehicle, Hecate, or Hecate–CG $\beta$  conjugate for 3 weeks. Hecate–CG $\beta$  conjugate treatment reduced the testicular and ovarian tumor burden ( $P < .05$ ), whereas a concomitant increase (testis;  $P < .05$ ) or no change (ovary) in tumor volumes occurred with Hecate treatment. A drop in serum progesterone, produced by the tumors, and an increase in LH levels occurred in Hecate–CG $\beta$  treated mice, in comparison with vehicle and Hecate groups, providing further support for the positive treatment response. Hecate–CG $\beta$  conjugate induced a rapid and cell-specific membrane permeabilization of LHR-expressing cells *in vitro*, suggesting a necrotic mode of cell death without activation of apoptosis. These results prove the principle that the Hecate–CG $\beta$  conjugate provides a novel specific lead into gonadal somatic cell cancer therapy by targeted destruction of LHR-expressing tumor cells.

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tumors are generally benign and account for about 2% of all testicular tumors, with malignancy occurring in about 10% of the cases. If metastases are found, chemotherapy is the treatment of choice because Leydig cell tumors are not radiosensitive [1]. Ovarian carcinoma is often called the “silent killer” because the disease remains usually undetectable until the advanced stage (II or IV) due to lack of diagnostic tests and absence of solid symptoms [3,4]. Among ovarian tumors, those in granulosa cells are rare, accounting for 3.0% to 7.6% of primary ovarian tumors. However, prospects of their treatment, in comparison to other ovarian cancers, are still poor [5,6]; tumor-related mortality rate is 37.3% [3,7], and approximately 80% of patients die of recurrent disease [7,8].

The major problems of cancer chemotherapies are related to required therapeutic concentrations of drugs, exerting undesirable effects on normal cells and causing severe side effects [9]. The development of targeted antitumor drug delivery systems has a great potential in enhancing chemotherapeutic efficacy and specificity. Most of the lytic peptides produced by insects, amphibians, and mammals have an amphipathic structure, and they preferentially bind and insert into negatively charged cell membranes [9]. In contrast to normal eukaryotic cells with low membrane potential, the prokaryotic and cancer cell membranes maintain a large membrane potential, and therefore many lytic peptides preferentially disrupt prokaryotic and cancer cell membranes rather than those of healthy eukaryotic cells [10].

To eradicate cancer cells and to reduce the toxicity of treatment toward normal tissues, we developed a novel approach for the treatment of endocrine tumors possessing luteinizing hormone receptors (LHRs). We synthesized the Hecate–chorionic gonadotropin  $\beta$  (Hecate–CG $\beta$ ) conjugate, a fusion protein of the 23–amino acid membrane lytic peptide

## Introduction

Early diagnosis and prevention of human gonadal tumors remain difficult, which makes them a fatal group of malignancies [1]. Although testicular tumors account for only 1% of all tumors in males, they are the most common malignancy in men between 15 and 34 years of age [2]. Leydig cell

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(Hecate) [11] and the 15–amino acid fragment of the human CG $\beta$  chain. The 81– to 95–amino acid region of CG $\beta$  composes the most potent region of the full-size CG protein with important [12] roles in receptor binding [13]. We hypothesized that the Hecate–CG $\beta$  conjugate could serve as an effective drug for the LHR expressed in gonadal tumors and possibly for some nongonadal tumors [14,15]. The affinity of the Hecate–CG $\beta$  conjugate to LHR by targeted destruction of LHR-expressing prostate, breast, ovarian, and testicular cancer cell lines [16,17], and in prostate PC-3, ovarian OVCAR-3, and breast MDA-MB-435S tumor xenografts implanted in nude mice *in vivo* has been demonstrated [16,18,19]. There are no existing reports wherein the therapeutic efficacy, endocrine, physiological, and pathophysiological consequences of Hecate or Hecate–CG $\beta$  conjugate treatment been evaluated, with the use of a transgenic (TG) preclinical model of tumorigenesis in a heritable immunocompetent model of disease.

In the present study, we investigated the therapeutic efficacy of the Hecate–CG $\beta$  conjugate to eradicate LHR-expressing tumor cells *in vivo* in a TG mouse model expressing the inhibin  $\alpha$ -subunit promoter (inh $\alpha$ )/Simian Virus 40 T-antigen (Tag) transgene and developing gonadal tumors [20,21], as well as monitored the subsequent endocrine effects of the treatment as an indication of the therapeutic effect. We also investigated *in vitro* the susceptibility of LHR-expressing tumor cells to Hecate–CG $\beta$  conjugate treatment and addressed the mechanisms underlying induced cell death, which has not yet been studied or reported.

## Materials and methods

### Cell Lines and Cultures

The murine Leydig tumor cell line (mLTC-1) (LHR-positive) [37] was cultured in Waymouth medium (Sigma Chemical Co., St. Louis, MO), supplemented with 9% heat-inactivated horse serum (Life Technologies, Paisley, Scotland, UK) and 4.5% heat-inactivated fetal calf serum (iFCS; Bioclear, Wokingham, Berks, UK) containing 0.1 g/l gentamicin (Gibco BRL, Gaithersburg, MD). Murine Leydig tumor BLT-1 (LHR-positive) [20], murine granulosa KK-1 (LHR-positive) [21], prostate cancer PC-3 (LHR-positive) [38], and colon carcinoma HT-29 (LHR-negative) [39] cells were maintained in DMEM/Ham's F-12 1:1 medium (Sigma), supplemented with 10% iFCS, 50 IU/l penicillin, and 0.5 g/l streptomycin (Sigma). The cells were allowed to grow on 9.6-cm–diameter Petri dishes (Greiner Labortechnik, Frickenhausen, Germany), or on 6- or 24-well plates (Greiner) to 70% to 80% confluency under a humidified atmosphere of 95% and 5% CO $_2$  at 37°C.

### Preparation of Drugs

Hecate and the Hecate–CG $\beta$  conjugate were synthesized and purified in the Peptide and Protein Laboratory, Department of Virology, Hartman Institute, University of Helsinki (Helsinki, Finland) as described earlier [29].

### Experimental Animals

TG mice of 5.5 months age, expressing the inh $\alpha$ /SV40 Tag transgene [40,22], all possessing macroscopic gonadal tumors, were selected for the experiment (7–10 mice per group). Wild-type (WT) littermate mice (C57Bl) were used as controls ( $n = 4$ –7 per group). For routine genotyping, PCR analyses were carried out using DNA extracts from tail biopsies, as previously described [21]. Mice were housed two to four per cage, after weaning at the age of 21 days, in a room with controlled light (12 hours of light, 12 hours of darkness) and temperature (21  $\pm$  1°C). The mice were specific pathogen-free and they were routinely screened for common mouse pathogens. Avertin anesthesia was used in surgical operations and postoperative analgesia (buprenorphine, 3  $\mu$ g/mouse, by intraperitoneal injections) was administered on a routine basis. The University of Turku Ethical Committee on Use and Care of Animals approved the animal experiments.

### Hecate and Hecate–CG $\beta$ Treatments

At the beginning of the experiment (at the age of 5.5 months), the gonadal size was assessed during laparotomy under general anesthesia, by measuring their length, depth, and width. The product of these three measurements (expressed in mm $^3$ ) was taken as approximation of the total gonadal volume, and the tumor volume burden was assessed as total gonadal volume per gram of body weight. Animals were treated with either vehicle or Hecate–CG $\beta$  conjugate (12 mg/kg) or Hecate (12 mg/kg) by intraperitoneal injections. The mice were injected once per week for three consecutive weeks, according to the earlier treatment protocols for *in vivo* treatment for nude mice xenografts [16,19,17]. Seven days following the last treatment, the mice were sacrificed by cervical dislocation, and blood was collected by cardiac puncture. Body, tumor, and different organ weights were recorded. Tissues were either snap-frozen in liquid nitrogen or fixed in Bouin's solution or 4% paraformaldehyde, dehydrated, and embedded in paraffin. Paraffin sections of 5  $\mu$ m thickness were stained with hematoxylin/eosin for histologic analysis.

### Northern Hybridization Analysis

Total RNA was isolated from WT and TG mice whole testis or ovary (C; Hecate– and Hecate–CG $\beta$  conjugate–treated groups) using the single-step guanidinium thiocyanate–phenol–chloroform extraction method [41]. Twenty micrograms of RNA per lane was resolved on 1.2% denaturing agarose gel and transferred onto Hybond-XL nylon membranes (Amersham International plc, Aylesbury, Bucks, UK). Membranes were prehybridized overnight at 65°C in a solution containing 5 $\times$  SSC, 5 $\times$  Denhardt's solution, 0.5% SDS, 50% formamide, and 5 g/l denatured calf thymus DNA. A complementary RNA probe for the rat LHR generated from a fragment of the LHR cDNA, spanning nucleotides 441 to 849 of its extracellular domain subcloned into the pGEM-4Z plasmid, was used for hybridization [12]. The [ $^{32}$ P]dUTP–labeled (800 Ci/mmol; Amersham International) probe was generated using a Riboprobe system II kit (Promega,

Madison, WI). The probes were purified with NickColumns (Pharmacia Biotech, Uppsala, Sweden). Hybridization was carried out at 65°C for 20 hours in the same prehybridization solution after the addition of labeled probe. After hybridization, the membranes were washed twice in 2 $\times$  SSC and 0.1% SDS at room temperature for 10 minutes, followed by two washes in 0.1 $\times$  SSC and 0.1% SDS at 65°C to remove most of the background. Finally, the membranes were exposed to Kodak X-ray films (Kodak XAR-5; Eastman Kodak, Rochester, NY) at -70°C for 4 to 7 days, or to a phosphor imager (Fujifilm BAS-5000; Fujifilm Ial, Tokyo, Japan) for 4 to 24 hours. The intensities of specific bands were quantified using the Tina software (Raytest, Staubenhardt, Germany) and related to those of the 28S ribosomal RNA in the gel stained with ethidium bromide. The molecular sizes of the mRNA species were estimated by comparison with mobilities of the 18S and 28S ribosomal RNA.

#### Hormone Measurements

LH concentration in sera was measured by a supersensitive immunofluorometric assay for rat LH (Delfia; Wallac Oy, Turku, Finland) as described earlier [40]. Progesterone was measured from diethyl ether extracts of the sera by RIA as described earlier [42,43]. We took additional serum samples ( $n = 7-8$ ) from 6.5-month-old tumor-bearing inh $\alpha$ /Tag male and female mice treated with vehicle as controls for hormone analyses.

#### Fluorescent Microscopy for PI Staining

To prove the preferential destruction of LHR-expressing cells and to determine the mechanisms involved in cell death caused by the Hecate-CG $\beta$  conjugate, we cocultured two cell lines, either murine Leydig mLTC-1 (LHR-positive) and human colon carcinoma HT-29 cells (LHR-negative) or murine granulosa KK-1 (LHR-positive) and human colon carcinoma HT-29 cells (LHR-negative) (data shown only for mLTC-1 and HT-29 cells), on the same glass well slides. Because the cells differed markedly in their shape and size, they were easy to distinguish. The cells were incubated for 24 hours in complete Waymouth medium, followed by a wash with 1 $\times$  PBS, and a further incubation in the presence or absence of 0.5  $\mu$ M Hecate or Hecate-CG $\beta$  conjugate for 15 minutes. Thereafter, the medium was removed and replaced with fresh medium containing PI, which does not enter cells with intact membranes, and thus differentiates between lysed and intact cells when studied by fluorescence microscopy. In the second set of experiments, we treated mLTC-1 and HT-29 cells for 30 minutes with concentrations of 0.5, 1, and 5  $\mu$ M Hecate. In the third set of experiments, we treated mLTC-1 cells with 0.5  $\mu$ M concentration of the Hecate-CG $\beta$  conjugate for 0, 15, and 30 minutes.

#### FACS Analysis

Cells from two Leydig tumor cell lines, mLTC-1 and BLT-1 [20], and a prostate cancer cell line, PC-3, were seeded into six-well plates in complete medium and incubated at 37°C in 5% CO $_2$  for 24 hours. Cells were washed once with PBS and

then incubated for the next 4 hours at increasing concentrations (0.1, 0.5, and 1  $\mu$ M) of Hecate or Hecate-CG $\beta$  conjugate. As an apoptotic positive control, we used 0.1% of hydrogen peroxide added to the culture media. Both adherent and floating cells were collected and stained with PI, and cellular DNA content was analyzed by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) as previously described [44,45].

#### Immunoblotting Analysis for Caspase-3 Activation

Western blotting analysis was used to determine whether caspase-3 was activated with an antibody recognizing the precursor and the active subunits. One day before stimulation, mLTC-1 cells were plated on six-well plates at a density of  $1.2 \times 10^5$  cells/well in 0.5 ml of complete Waymouth medium. The cells were washed once with PBS and then incubated for 60 minutes at 0.5, 1, and 5  $\mu$ M Hecate or Hecate-CG $\beta$  conjugate. Total cell lysates were prepared as described previously [46] and subjected to electrophoresis through a 12.5% SDS-PAGE gel (60  $\mu$ g protein/well). After electrophoresis, proteins were electrotransferred to nitrocellulose membranes. After blocking the membrane, it was incubated with the primary rabbit monoclonal antibody (Cell Signaling, Beverly, MA). Signals were visualized using ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) and finally exposed to X-ray film.

#### Caspase Inhibitor Z-VAD.fmk

Cells were seeded into six-well plates at a density of  $2 \times 10^5$  cells/well. Twenty-four hours later, the medium was replaced with new medium containing 20  $\mu$ M pan-caspase inhibitor Z-VAD.fmk (Calbiochem, Nottingham, UK) and incubated for 1 hour. Hecate or Hecate-CG $\beta$  conjugate was then added at concentrations of 0.1, 0.5, and 1  $\mu$ M and incubated overnight. Incubation with the conjugate was stopped after 16 hours. A colorimetric MTT assay was then performed to measure cell survival [47]. We used 0.1% H $_2$ O $_2$  as the positive control for apoptosis. Viability in the treated cells was expressed as a percentage of controls. The untreated controls were assigned a value of 100%.

#### Statistical Analysis

Statistical ANOVA paired  $t$  test analyses of variance were carried out using a StatView program for Windows (version 5.0.1) (SAS Institute, Inc., Cary, NC). All data are presented as the mean  $\pm$  SEM.

## Results

#### Hecate-CG $\beta$ Treatment Reduced Tumor Volume

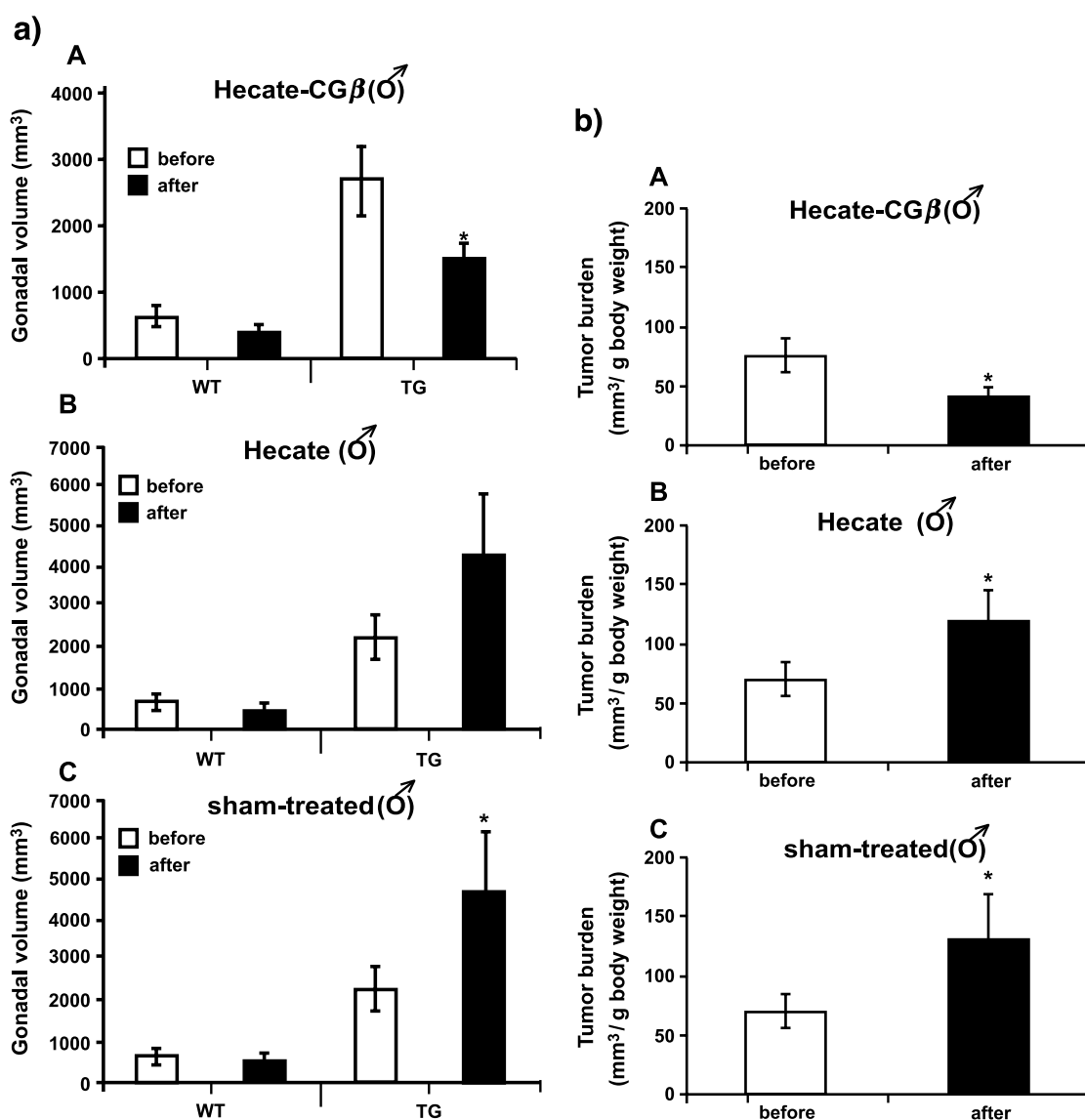
We were able to achieve an antineoplastic effect of the Hecate-CG $\beta$  conjugate on the inh $\alpha$ /Tag TG mice. Here we have used the same treatment strategy protocols used earlier and established for *in vivo* xenograft treatments [16,18,19]. Following 1-month treatment, total testicular tumor volume decreased by an average of 58% (Figure 1a;

$P < .05$ ; compared to the volume before treatment) and that of the ovaries by 36% (Figure 1c). As the age-related tumor progression rate was variable between animals, which has been observed in the *inh $\alpha$ /Tag* and *Tag* mice before [22,20,21,23], we decided to show the tumor burden, too. The gonadal tumor burden (i.e., tumor volume/g body weight) after Hecate-CG $\beta$  conjugate treatment in both sexes decreased significantly in comparison with that analyzed before treatment (Figure 1, b and d;  $P < .05$ ). In the Hecate-treated males, tumors grew steadily as in the sham-treated control groups (Figure 1, a and b); but in females, the ovarian tumor volumes did not change significantly (Figure 1, c and d). Hecate-CG $\beta$  conjugate and Hecate treatments showed no significant influence on gonadal volume in the WT mice group (Figure 1, a and c).

There were no statistically significant differences between the body weights among comparable groups (like Hecate- or conjugate-treated, before and after the WT and TG groups).

#### Hecate-CG $\beta$ Treatment Was Specific Toward LHR-Possessing Cells

To assess whether the Hecate-CG $\beta$  treatment reduced the LHR mRNA levels in comparison to Hecate- and sham-treated TG control groups, we made a quantitative evaluation from RNA extracts of the total testes and ovaries of TG mice. Northern hybridization revealed that there was a concomitant decrease in LHR mRNA expression in the Hecate-CG $\beta$ -treated testes and ovaries ( $P < .01$  and  $P < .05$ ; versus Hecate-treated groups and/or the sham-treated TG control



**Figure 1.** Total gonadal volumes of male (a) and female (c) wild-type (WT) control littermate mice and *inh $\alpha$ /Tag* transgenic (TG) mice, before and after 3 weeks of Hecate-CG $\beta$  conjugate (Panels A), Hecate (Panels B), or sham treatment (Panel C). (b and d) Tumor burden volumes (tumor volume/g body weight). The values are mean  $\pm$  SEM of both gonads. \* $P < .05$ , Hecate, Hecate-CG $\beta$  conjugate, or sham-treated gonadal volume after treatment versus before treatment. WT, wild-type control littermate mice; TG, transgenic mice expressing the *inhibin $\alpha$*  Simian Virus 40 T-antigen (*inh $\alpha$ /Tag*).

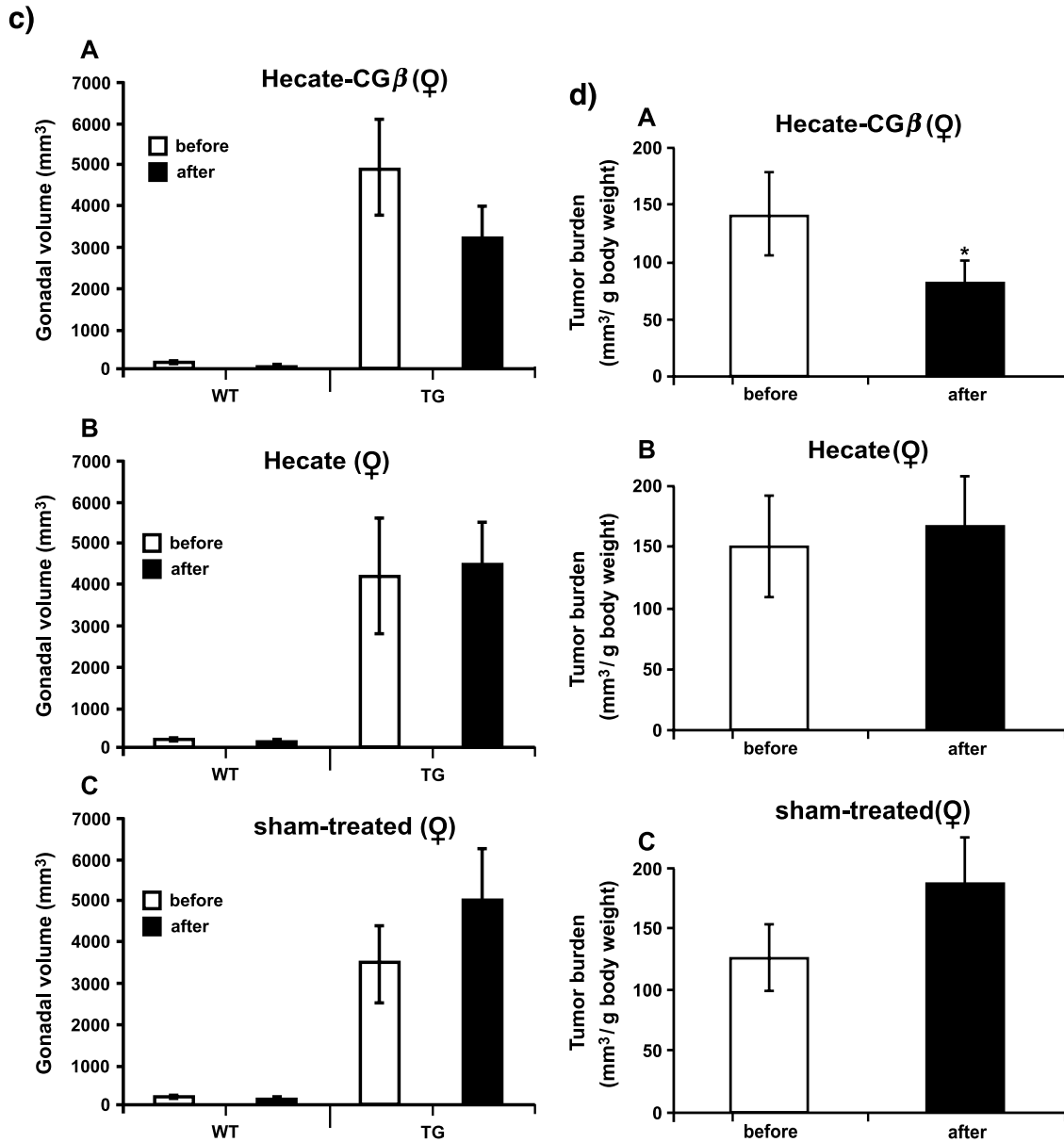


Figure 1. (continued)

group) (Figure 2), which confirmed the LHR-specific destruction effects of Hecate-CG $\beta$  and was in line with the reduced tumor volume data (Figure 1).

#### Endocrine Consequences of Treatment

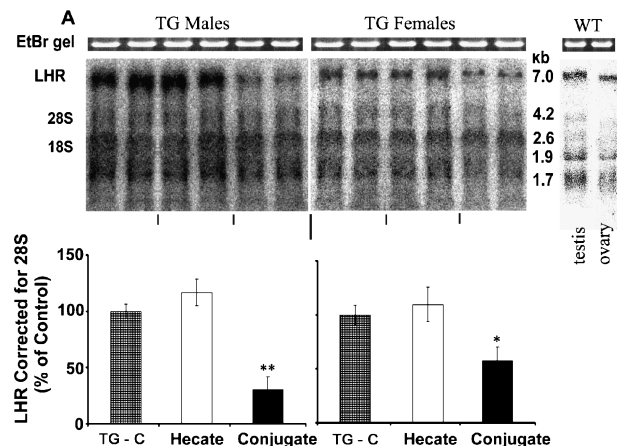
We have earlier shown that the serum progesterone levels rise significantly and those of gonadotropins drop along with tumorigenesis (6-month-old TG mice) in comparison with WT control littermates [24,25]. After 3 weeks of treatment with the Hecate-CG $\beta$  conjugate, serum progesterone concentration decreased significantly both in females and males (Figure 3A;  $P < .05$ ; versus Hecate-treated groups and the sham-treated TG control group) and serum LH concentration increased in the Hecate-CG $\beta$  conjugate-treated groups (Figure 3B;  $P < .01$  to  $P < .05$ ; versus

Hecate-treated groups and the sham-treated TG control group). The hormonal values thus allowed us to monitor the positive treatment effects of the Hecate-CG $\beta$  conjugate.

#### Histopathologic Analysis Demonstrates Antitumoral Effect of Hecate-CG $\beta$ Conjugate Treatment

Histopathologic analysis showed that Hecate-CG $\beta$  conjugate treatment of TG mice induced a definite degradation of testicular and ovarian tumor mass (more marked in testis). In many parts of the histologic tumor sections, we found clear changes in tumor tissues, apparently induced by the treatment. Especially in testicular sections, a high number of neoplastic Leydig cells were destroyed, and the tumor tissues apparently contained necrotic cells (Figure 4, B and D). In contrast, in tissue sections collected from TG mice





**Figure 2.** Northern hybridization analysis of LHR mRNA expression in Hecate and Hecate-CG $\beta$  conjugate-treated or sham-treated testicular and ovarian tissues of *inh $\alpha$ /Tag* transgenic (TG) males and females. Each lane contains 20 mg of total RNA. The migration of the 28S and 18S rRNA are shown on the left side of the LHR panel. The sizes of the different LHR mRNA splice variants (in kb) are presented on the right. Two lanes for each type of sample are depicted. One lane of WT male and female gonad mRNA expression is shown on the right as a positive control for LHR mRNA. The upper panel shows on the top the ethidium bromide (EtBr) staining of the 28S rRNA for RNA loading control. The middle panel shows Northern hybridization for LHR mRNA. The lower panel shows the densitometric quantification of the longest (7.0 kb) LHR mRNA splice variant (open bar for Hecate and filled bars for sham-treated and Hecate-CG $\beta$ -treated) in arbitrary densitometric units (mean of TG sham-treated testis and ovaries regarded as 100%) corrected for intensity of the 28S rRNA band. Each bar represents the mean  $\pm$  SEM of three independent experiments in duplicates. \*\* $P < .01$ , \* $P < .05$  ( $P < .01$  and  $P < .05$ ; Hecate-CG $\beta$ -treated versus Hecate-treated and/or sham-treated TG control group). TG, transgenic mice expressing the inhibin $\alpha$  Simian Virus 40 T-antigen (*inh $\alpha$ /Tag*); TG-C, sham-treated TG mice.

treated with Hecate as well as in sham-treated TG mice (data not shown), cell death appeared quite sporadically and multiple mitoses were seen in the tumors (Figure 4, A and C). The histologic results supported the data of reduced tumor volumes in Figure 1. We studied the cross-sections of several other organs (lungs, pancreas, liver, spleen, adrenals, and uterus) for posttreatment side effects or metastases. Only in two males and one female treated with Hecate or vehicle were lung metastases found, but never in Hecate-CG $\beta$  conjugate-treated groups. Histologic study revealed no apparent effects of the treatments in extra-gonadal organs (data not shown).

#### Mode of Antitumor Effect and Specificity of Selective Cell Death

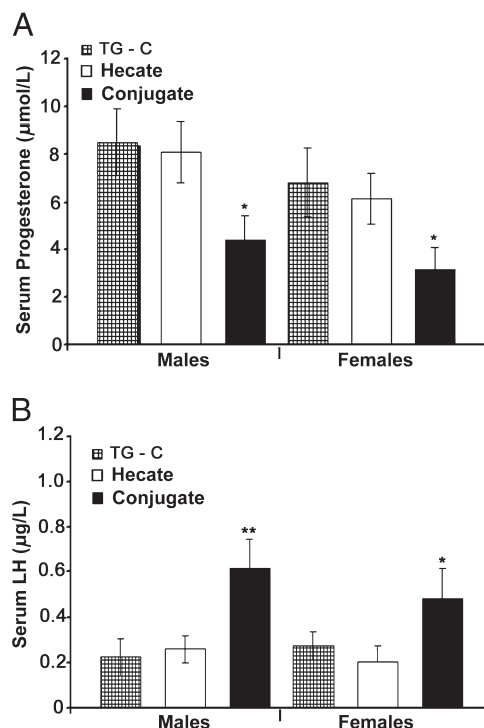
The preferential killing of LHR-expressing mLTC-1 Leydig cells, but not of LHR-negative colon carcinoma HT-29 cells, was clearly shown with the propidium iodide (PI) exclusion experiment (Figure 5A). Nuclear PI staining showed that 15 minutes of 0.5  $\mu$ M Hecate-CG $\beta$  conjugate treatment induced rapid permeabilization of the cell membrane and killing of LHR-positive mLTC-1 cells, but not of HT-29 cells (Figure 5A). Hecate treatment for 30 minutes at 1  $\mu$ M concentration killed HT-29 cells only; but at 5  $\mu$ M concentration, it killed both HT-29 and mLTC-1 cells (vs 0.5  $\mu$ M

Hecate-CG $\beta$  conjugate, killing mLTC-1 cells; Figure 5A) with similar efficacy, regardless of the LHR expression (Figure 5B). At 0.5  $\mu$ M concentration, Hecate did not have any effects on either cell line (Figure 5B). It has never been shown earlier in such a simple but trivial way that the Hecate-CG $\beta$  conjugate selectively kills cells possessing LHR but spares cells without LHR.

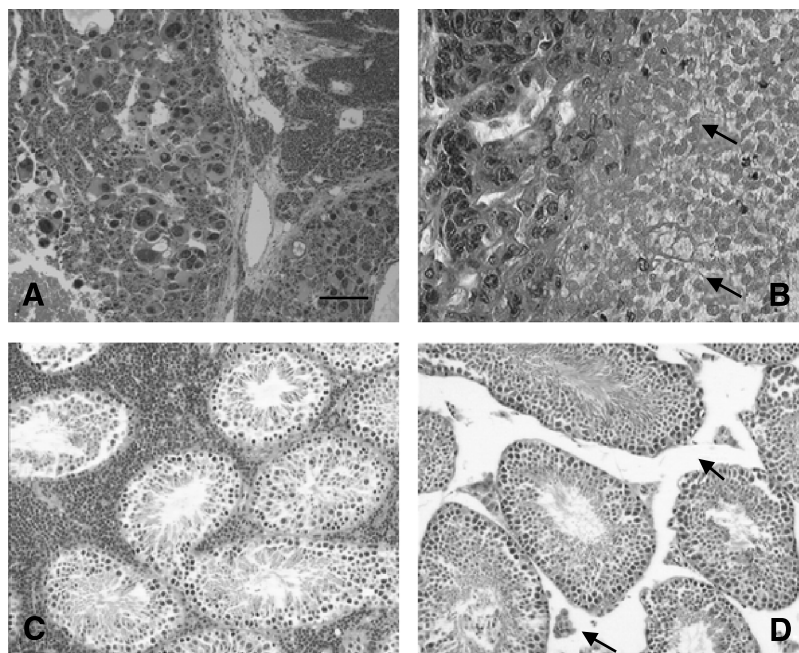
We analyzed further whether the molecular mode of cell death induced by the Hecate-CG $\beta$  conjugate could be by necrosis. Light microscopic analysis revealed that the moderate dose of 0.5  $\mu$ M Hecate-CG $\beta$  conjugate for 30 minutes induced swelling of the mLTC-1 cells (Figure 5C), indicating that the cells died as a result of acute injury, swelling, and bursting, which strongly suggest necrosis [26,27].

#### Further Studies on Molecular Mechanisms of the Selective Death of LHR-Expressing Cells Induced by the Hecate-CG $\beta$ Conjugate

**Flow cytometric analysis.** The cells from all the three cell lines (data shown only for mLTC-1 cells) treated with Hecate or Hecate-CG $\beta$  conjugate were lysed, cell nuclei were stained with PI, and the percentage of cells undergoing apoptosis by nuclear fragmentation was determined. The percentage of cells undergoing apoptosis in the presence of H<sub>2</sub>O<sub>2</sub> was 62.4%, 53.6%, and 88.8% for mLTC-1, BLT-1,



**Figure 3.** Serum progesterone (A) and LH (B) concentrations in *inh $\alpha$ /Tag* transgenic female and male mice after the 3-week treatment with either Hecate, Hecate-CG $\beta$  conjugate, or sham (TG-C). \*\* $P < .01$ , \* $P < .05$  ( $P < .01$  and  $P < .05$ ; Hecate-CG $\beta$ -treated versus Hecate-treated and/or sham-treated TG-C control group). TG, transgenic mice expressing the inhibin $\alpha$  Simian Virus 40 T-antigen (*inh $\alpha$ /Tag*); TG-C, sham-treated TG mice.



**Figure 4.** Histologic pictures of hematoxylin/eosin-stained ovarian granulosa (A and B) and testicular Leydig cell (C and D) tumors of *inh $\alpha$ /Tag* mice treated with Hecate (A and C) or Hecate-CG $\beta$  conjugate for three consecutive weeks. Arrows indicate that the interstitial tissue (B and D) has dramatically shrunken after Hecate-CG $\beta$  conjugate treatment (the bar in panel A is 40  $\mu$ m; panels B–D have the same magnification as panel A).

and PC-3 cells, respectively (Figure 6). All three cell lines challenged with increasing concentrations of Hecate-CG $\beta$  conjugate or Hecate (0.1, 0.5, and 1  $\mu$ M) for 4 hours showed lack of nuclear fragmentation (Figure 6).

**Analysis of caspase-3 activation.** Incubation of mLTC-1 cells with Hecate-CG $\beta$  conjugate or Hecate at 0.5  $\mu$ M concentration for 90 minutes did not induce the activation of caspase-3 when analyzed by Western blot analysis (Figure 7). This treatment caused a decrease in cell viability but no active form (17 kDa) of caspase-3 could be detected (Figure 7). These data provide strong evidence that the cell death occurred without the proteolysis of procaspase.

Next, to check whether any activation of the apoptotic pathways had taken place while causing cell death by Hecate or Hecate-CG $\beta$  conjugate, we used the pan-caspase inhibitor to pretreat the BLT-1 and mLTC-1 Leydig tumor cells. Both BLT-1 and mLTC-1 cell viability showed similar decreased levels after treatment with the Hecate-CG $\beta$  conjugate, as it was caused without the inhibitor pretreatment levels (results not shown). The minimum concentration of the Hecate-CG $\beta$  conjugate leading to 50% cell death (EC<sub>50</sub>) was 0.1  $\mu$ M for mLTC-1 cells. The same concentration of the conjugate decreased the viability of BLT-1 cells until 40%. In the presence of 0.5 to 1  $\mu$ M concentration of the conjugate and pretreatment with the Z-VAD.fmk inhibitor, cell viability oscillated between 50% and 40% for mLTC-1 cells and between 35% and 30% for BLT-1 cells (results not shown). The presence or absence of the Z-VAD.fmk inhibitor did not significantly change Leydig tumor cell viability after the treatment of the Hecate-CG $\beta$  conjugate, but in the positive control for

apoptosis with 0.1% of H<sub>2</sub>O<sub>2</sub>, the presence of Z-VAD.fmk was able to block apoptosis significantly (50%) (results not shown). These results further proved that the mode of cell death caused by the Hecate or Hecate-CG $\beta$  conjugate was not apoptosis, but necrosis, as shown in Figure 5C.

## Discussion

In the present study, we took advantage of the established *inh $\alpha$ /Tag* TG mouse model of gonadal somatic cell tumorigenesis [20,28,21,24,23], where the pathophysiological and endocrine responses induced by tumorigenesis are well-known. This information allowed us to gain insight into the specific endocrine and paracrine consequences of the Hecate and Hecate-CG $\beta$  conjugate treatments. Here, we were able to demonstrate in TG mice that the targeted treatment with the Hecate-CG $\beta$  conjugate, apparently through binding to LHR, brought about effective blockage and/or reduction of tumor growth. Hecate-CG $\beta$  conjugate treatment was able to reduce the total gonadal tumor volumes of *inh $\alpha$ /Tag* mice, without causing any noticeable side effects.

It has been previously shown that the Hecate-CG $\beta$  conjugate is able to bind specifically to target cell membrane LHR [29], including LHR-expressing tumor cells [16,17]. The higher killing efficiency of the Hecate-CG $\beta$  conjugate of Leydig *versus* granulosa cells may be due to a higher level of LHR expression in the former cell types. In accordance, the granulosa (KK-1) and Leydig (BLT-1) cell lines derived from the *inh $\alpha$ /Tag* mouse gonadal tumors expressed 9870 vs 36,000 binding sites per cell, respectively [20,21]. The

Leydig cell tumors with a higher number of LHR per cell were more susceptible to the destructive action of the Hecate-CG $\beta$  conjugate, as shown by histopathologic analysis. We did not observe any drug-related alterations in other organs like the liver, kidney, or uterus. Besides the LHR expression of the tumor cells, electrostatic interactions between negatively charged tumor cell membranes and nontumor cells may explain the specificity of the action [30]. Some *in vivo* studies have shown that the specific anticancer activities of some native and synthetic cationic lytic peptides (e.g., Hecate) occurred only when they were conjugated to homing domains and were quite limited [30,31]. Hecate might be cytotoxic to prokaryotic [11] and eukaryotic [17] cells, but only at much higher concentrations, and then to cell types regardless of their LHR expression [29], as also shown in this study.

In the inh $\alpha$ /Tag TG mice, the onset of tumor formation paralleled increased serum progesterone and decreased gonadotropin concentrations, with the former apparently originating from an increased number of progesterone-secreting tumor cells in the ovary [28,24,25], indicating that the tumors were endocrinologically active and able to exert enhanced negative feedback effects on pituitary function [24,25,23]. The steroidogenesis of Leydig BLT-1 and mLTC-1 cells was partly impaired because it did not proceed beyond progesterone to testosterone [20]. However, progesterone was also able to exert negative feedback actions on gonadotropin secretion [24,25], which explains the suppression of their secretion in male mice. Hence, if the targeted antitumor therapy with the Hecate-CG $\beta$  conjugate kills Leydig and granulosa tumor cells, then the elevated level of serum progesterone would decrease and serum LH concentration would increase. Indeed, after 3 weeks of treatment with the Hecate-CG $\beta$  conjugate, the progesterone concentration was significantly decreased in the inh $\alpha$ /Tag mice and serum LH concentrations were significantly elevated, as compared with the respective levels of untreated tumor mice and those receiving Hecate. This was considered a distinct sign of endocrine recovery from the hormonally active tumorigenesis under the Hecate-CG $\beta$  conjugate treatment.

We finally wanted to investigate the molecular mechanisms underlying the mode of action of the Hecate-CG $\beta$  conjugate, namely the mechanisms involved in targeted cell death. The cytotoxic effect of most antitumor agents de-

pends on the induction of apoptosis in susceptible tumor cells [32]. Apoptotic cell death is characterized by a series of specific events including the activation of the caspase cascade, cell shrinkage, cell membrane blebbing, and condensation and fragmentation of chromatin [33]. There is evidence that the sensitivity of various tumor types to current therapeutic methods depends on the activation of multiple apoptosis-regulatory proteins [33,31,34]. There is a preliminary report showing the involvement of phospholipase PKC rather than PKA (PKC increased the sensitivity to the drug) in breast cancer cell lysis by lytic peptide hecate [19], although it has been also shown that PKC activation can be antiapoptotic, too [35,36]. In this study, two types of cocultured cell lines with or without endogenous LHR treated with Hecate-CG $\beta$  proved the high specificity for the LHR-expressing cells only, which has not been shown earlier so precisely. The chain of events showed that the binding of the Hecate-CG $\beta$  conjugate to the LHR and the rapid (within 15 minutes of PI administration) permeabilization and perturbation of cell membrane were the cause of cell death. It seems that once the membrane layer integrity is disturbed, which happens rapidly within minutes, the transmembrane electrochemical potential collapses and cell death occurs. Cell viability measurements and fluorescence microscopic observations verified that the cells died as a result of injury, swelling, and bursting, suggesting signs of mechanisms of induction of necrosis, which also has never been shown before. All attempts to demonstrate apoptosis as a result of the treatments yielded negative results.

Taken together, the present data provided strong evidence for a rapid and specific killing mechanism by the Hecate-CG $\beta$  conjugate toward LHR-expressing gonadal tumor cells through necrotic plasma membrane disruption. Cancer cells commonly have impaired/deleterious or mutated genes involved in apoptosis, making them often apoptosis-resistant. Therefore, the specific targeted necrosis induced by the Hecate-CG $\beta$  conjugate for cancer cells may offer an advantage and assure that the neoplastic cells would not develop drug resistance to destructive mechanism. Our *in vivo* results demonstrate that the targeted therapy of gonadal somatic cell tumors expressing LHR by the Hecate-CG $\beta$  conjugate is potentially useful and worthy of further testing, with potential for future human trials.

**Figure 5.** Fluorescent and light microscopic analysis of cocultured Leydig tumor mLTC-1 (LHR-positive, bigger cells) and colon carcinoma HT-29 (LHR-negative, smaller cells) (marked with arrows). (a) Pretreated with 0.5  $\mu$ M Hecate-CG $\beta$  conjugate for 15 minutes in the presence of nonpermeable PI in the media. The left panels (A and B) showed the light microscopic pictures of two different cell mixtures. The upper and lower middle darker field panels (C and D) showed PI fluorescent microscopy. The cell membranes of the mLTC-1 cells were preferentially perturbed by the Hecate-CG $\beta$  conjugate. Panels E and F are merged pictures of light and PI fluorescent microscopy (the bar in panel A is 40  $\mu$ m; panels B-F have the same magnification as panel A). PI, propidium iodide. (b) Pretreated for 30 minutes with an increasing dose of Hecate: (A and D) 0.5  $\mu$ M, (B and E) 1  $\mu$ M, and (C and F) 5  $\mu$ M Hecate in the presence of nonpermeable PI in the media. The left panels (A-C) show the light microscopy pictures. The right darker panels (D-F) show PI fluorescent microscopy. Hecate killed both mLTC-1 cells and HT-29 cells with the highest (5  $\mu$ M) concentration used not depending on their LHR content. A concentration of 1  $\mu$ M Hecate killed only HT-29 cells (the bar in panel A is 40  $\mu$ m; panels B-F have the same magnification as panel A). PI, propidium iodide. (c) Light microscopic picture of a single mLTC-1 Leydig tumor cell incubated in 0.5  $\mu$ M Hecate-CG $\beta$  conjugate for 0, 15, and 30 minutes. Cell swelling could be observed at 30 minutes (C), which strongly suggests necrosis as the mode of cell death (the bar in the panel A is 40  $\mu$ m; panels B-C have the same magnification as panel A).



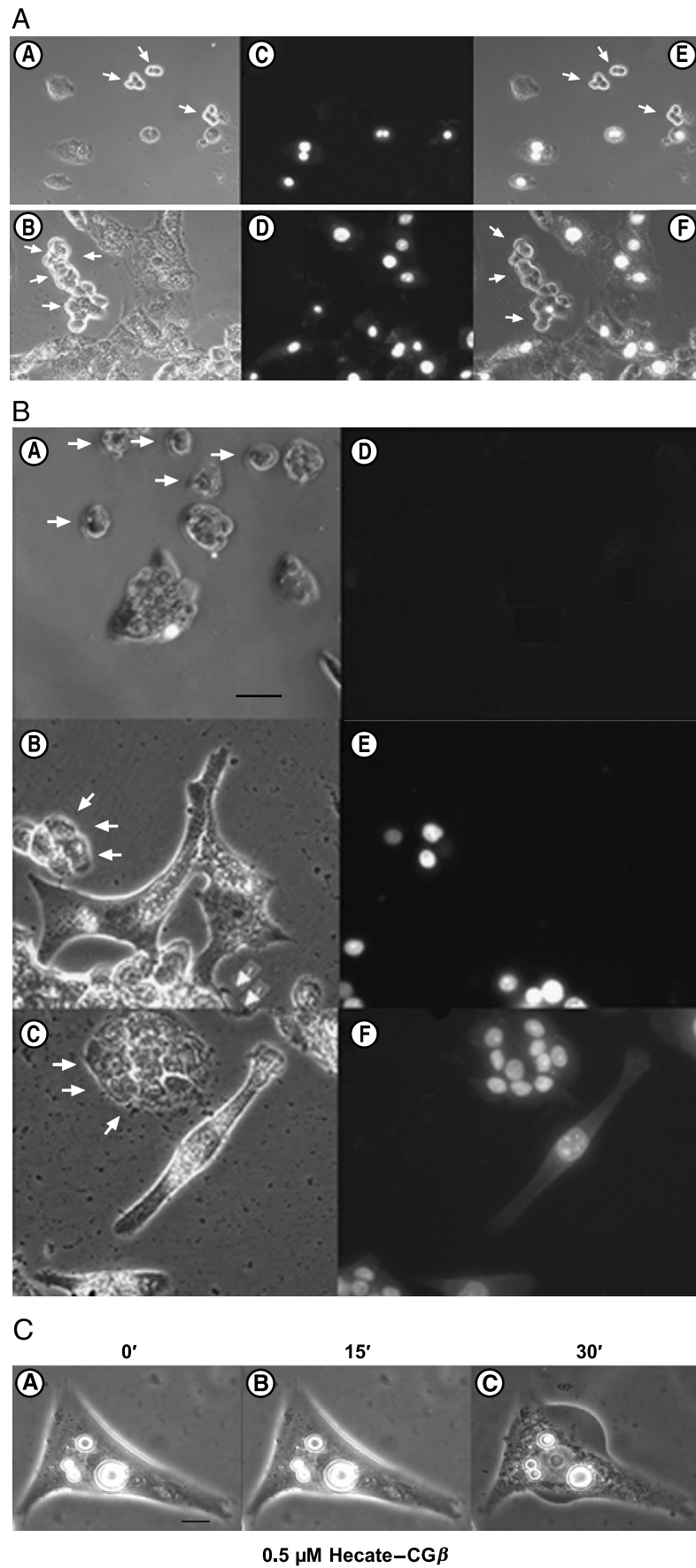
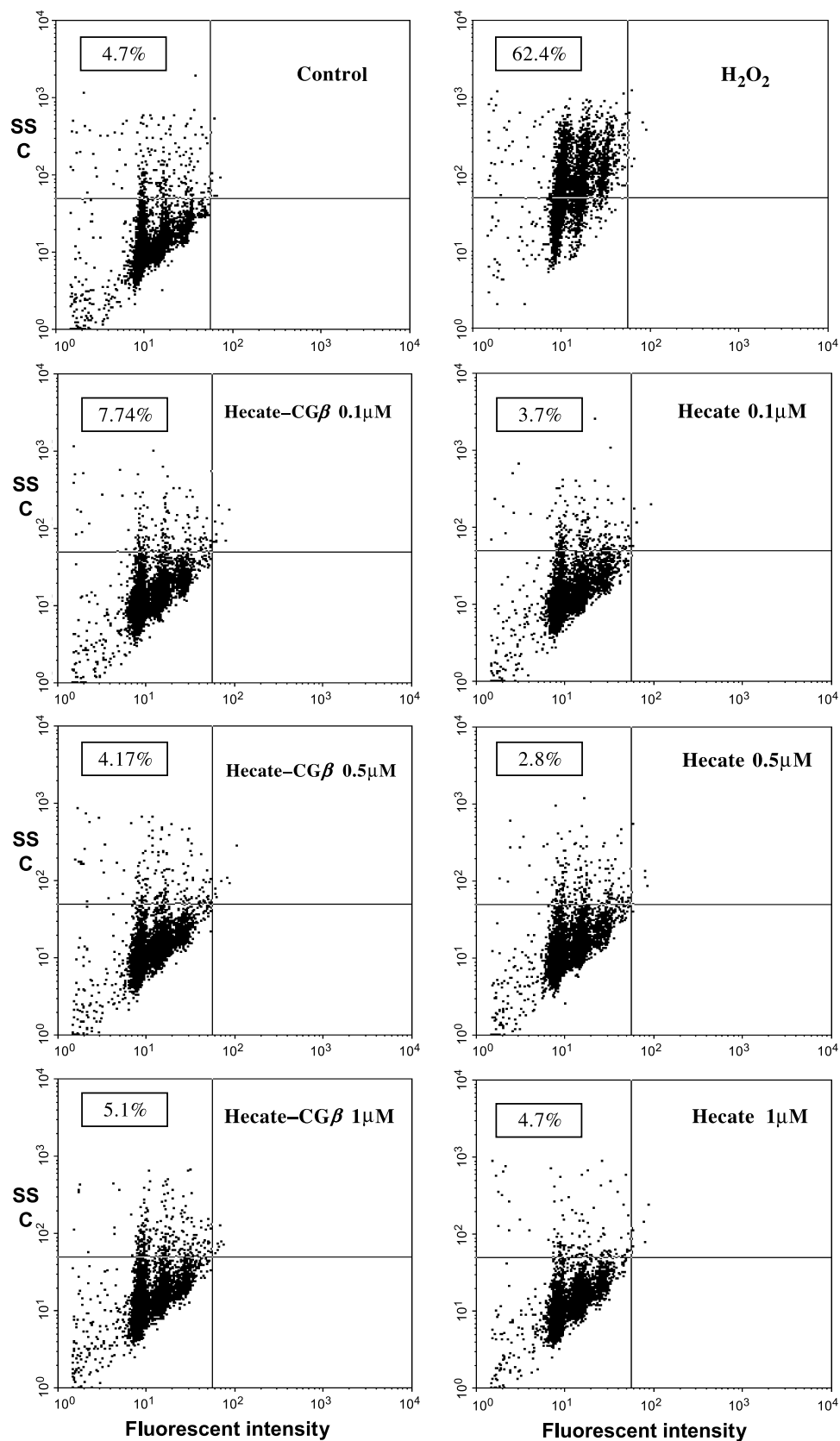
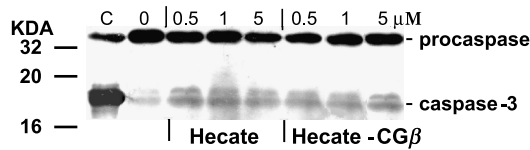


Figure 5.



**Figure 6.** Flow cytometric analysis of mLTC-1 cells treated for 4 hours with concentrations of 0.1, 0.5, and 1  $\mu$ M Hecate-CG $\beta$  conjugate or Hecate. Cells treated with 0.1% H<sub>2</sub>O<sub>2</sub> for 4 hours were used as positive apoptotic controls. After incubation, cells were lysed in a hypotonic solution containing nonpermeable PI and analyzed by FACS (FACSCalibur flow cytometer; Becton Dickinson). Neither Hecate-CG $\beta$  conjugate nor Hecate treatment induced nuclear fragmentation in mLTC-1 Leydig cells. The numbers on the top left quadrant of the panels represent the percentage of apoptotic cells. The data are representative of three separate experiments with similar results.



**Figure 7.** Western blot analysis of caspase-3. mLTC-1 cells were treated with 0.5, 1, and 5  $\mu$ M of either Hecate or Hecate-CG $\beta$  conjugate for 60 minutes. Adherent and detached cells were assayed for cleaved caspase-3. No detectable active caspase-3 activity in Hecate- and Hecate-CG $\beta$ -treated Leydig tumor mLTC-1 cells could be observed. "C"—mLTC-1 cells treated with 0.1% H<sub>2</sub>O<sub>2</sub> showing cleaved caspase-3, positive control for apoptosis; on the left side, the molecular marker sizes are shown in kilodaltons.

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